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By FRANK H. WILEY.

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIRE-
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THE ACTION OF NITROUS ACID ON CASEIN. II.

By FRANK H. WILEY AND HOWARD B. LEWIS.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

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When nitrous acid acts upon proteins, nitrogen is liberated and the resulting products, known as deaminized proteins, show properties differing in important respects from the original proteins from which they are derived (1). The nitrogen liberated in the reaction is usually considered to originate from the reaction between the nitrous acid and the ϵ -amino group of lysine (2). Other reactions which involve the characteristic groups of amino acids other than lysine have also been suggested. Thus, according to some investigators, tyrosine is so altered in the process of deamination that deaminized proteins give no Millon's test nor can tyrosine be isolated after hydrolysis of the protein (3). No adequate explanation has been given for the yellow or yellow-brown color characteristic of deaminized proteins.

In view of the development of improved methods of analysis of proteins within recent years, it seemed that application of these methods to the analysis of deaminized proteins might yield valuable data, data which would extend earlier work from this laboratory (1, 3).

For the study of the products of deamination, casein was selected as a convenient material since this protein is easily obtainable in a purified form and yields after deamination a product which is insoluble and hence easily purified. We have studied the properties and composition of deaminized caseins in whose preparation different temperatures were employed in the hope that at lower temperatures, the possibility of secondary reactions might be more limited. Our attention has been directed chiefly to the distribution of nitrogen as determined by the Van Slyke procedure, to the content of tyrosine and tryptophane, and to the content of the basic amino acids.

The method used for the preparation of the deaminized casein was a modification of the procedure of Skraup, as previously outlined by Dunn and Lewis (1). All the factors except the temperature of the reaction mixture during the period of the addition of the sodium nitrite were kept as nearly constant as possible. Products of deamination at varying temperatures, 0, 20, 40, and 60°, were prepared and analyzed. After the reactions of deamination had proceeded at the temperature indicated for 1½ hours, the mixture was allowed to stand for 18 hours, in the ice box in the case of the material deaminized at 0°, and at room temperature in all other experiments.

Since it was possible that contact with the acetic acid in which the casein was dissolved might alter the molecule independently of the action of the nitrous acid, one experiment was carried out in which casein was dissolved in acetic acid and the acid subsequently neutralized with sodium hydroxide. This procedure was carried out under exactly the same conditions as were used in the preparation of the casein deaminized at 20°, an amount of sodium hydroxide equivalent to the acetic acid being added instead of the sodium nitrite. The reaction after the addition of the alkali was close to the isoelectric point of casein and the protein was precipitated. This preparation, washed and dried in the usual manner, will be designated as "denaturized" casein merely for convenience in order to distinguish it from the original untreated casein.

The color of the deaminized proteins varied with the temperature of deamination, the products obtained at lower temperatures being light yellow in color while those obtained at higher temperatures were brownish. The surface of the deaminized casein became dark when exposed to light. The yield as in earlier experiments (1) was between 90 and 95 per cent of the casein used.

Not only were the deaminized caseins analyzed, but also the original samples of casein, which were used in the preparation of the deaminized proteins. Moisture and ash determinations were made on all samples and all the analytical data reported are calculated on an ash-free moisture-free basis.

Partition of Nitrogen by the Method of Van Slyke.

The method originally proposed by Van Slyke for the analysis of proteins by the determination of characteristic groups is subject to many errors such as those due to the destruction of cystine in the acid hydrolysis, to the incomplete precipitation of the basic amino acids by phosphotungstic acid, and to the precipitation of amino acids other than the basic amino acids by the phosphotungstic acid. However, it was believed that in the comparison of two proteins as similar in nature as casein and deaminized casein, and in the comparison of deaminized caseins prepared at various temperatures, these errors would be of approximately the same magnitude and that some indication of the effects of nitrous acid on the protein molecule would be given.

Eight samples of deaminized casein, prepared under slightly different conditions, two of casein and one of "denaturized" casein were analyzed. Each analysis of those presented in Table I is the average of determinations carried out in triplicate. The method as proposed by Van Slyke was used without essential modification throughout except that the apparatus described by Koehler (4) was used for the determination of the arginine nitrogen of the bases.

"Denaturized" casein showed, within the limits of the experimental error of the method, the same analysis as casein, thus indicating that acetic acid alone had practically no effect on the protein molecule as far as could be determined by this method.

The total nitrogen of the deaminized casein was in all cases slightly less than that of the casein from which it was prepared as was also the total amino nitrogen after hydrolysis. There appeared to be a slight decrease of the arginine nitrogen of the bases, destruction of arginine increasing with the temperature of deamination, shown most significantly in the sample deaminized at 60° (Deaminized Casein VII, Table I). This was not unexpected since it has been shown by Sekine (5) and others (6, 7) that arginine may lose more than one-quarter (an amount corresponding to the nitrogen present as the α -amino group) of its nitrogen, when allowed to react with nitrous acid for long periods of time.

The results obtained for the histidine content of casein were much lower than those usually reported for casein by this method

(4.24 to 6.55 per cent). There appeared to be an increase in the histidine content of most of the deaminized caseins. This increase, however, was probably within the limits of error of the method since histidine was determined indirectly and, in our hands, duplicate determination frequently showed marked variations. In a later part of this paper, in which the isolation and more direct determination of histidine are reported, it will be

TABLE I.

Analyses of Casein, Deaminized Casein, and "Denaturized" Casein.

Total N is reported in per cent of N in protein. The remainder of the figures are in terms of per cent of the total N present.

Protein analyzed.....	Casein II.	Deaminized casein No.*				"De- natur- ized" casein.
		V	VI	VII	VIII	
Total N.....	14.95	14.72	14.76	14.61	14.73	14.93
Amino ".....	76.59	71.69	69.62	69.45	69.81	76.25
Amide ".....	9.98	10.65	11.24	11.34	11.26	10.32
Melanin N.....	1.32	1.23	1.93	2.05	2.00	1.71
Total N of bases.....	20.63	17.42	15.49	13.00	15.63	20.81
Amino N of bases.....	13.84	8.93	7.84	5.76	7.04	13.87
Arginine N.....	7.63	8.17	7.16	6.37	7.08	7.80
Histidine N.....	1.72	3.54	3.31	3.70	5.04	1.64
Cystine N.....	0.59	1.60	1.55	1.55	0.83	0.65
Lysine N.....	10.70	4.12	3.47	1.39	2.68	10.73
Total N of filtrate.....	66.36	70.05	71.10	73.68	70.12	66.51
Amino N of filtrate.....	61.20	63.14	64.20	63.23	61.34	60.35
Non-amino N of filtrate.....	5.16	6.54	6.90	10.45	8.78	6.16
Total N recovered.....	98.30	99.36	98.77	100.06	99.01	99.35

* In the preparation of Deaminized Caseins V to VIII, the temperatures of the initial stage of the deamination were 0, 20, 60, and 20° respectively.

shown that deaminized casein actually contained less histidine than did the casein used in the deamination.

The lysine content of the solution of the bases from deaminized caseins was markedly decreased. With the ϵ -amino group replaced by a hydroxy group, lysine would presumably lose its basic properties, and the product of deamination corresponding to lysine would be expected to appear in the filtrate from the bases. This should result in increase in the amino nitrogen of that fraction equivalent to one-half of the lysine nitrogen.

Although in our analytical results an increase in the total nitrogen of the filtrate was evident in all cases after deamination, the greater part of this increased nitrogen was shown to be present in the non-amino fraction in most cases.

The results indicate that the destruction of lysine was a direct function of the temperature of deamination. Thus the proteins deaminized at 0° showed a lysine nitrogen content of 5.83 and 4.12 per cent of the total nitrogen; at 20°, 3.78, 3.47, and 2.68 per cent; at 40°, 3.31 per cent; and at 60°, 2.33 and 1.39 per cent. These results which do not indicate a complete destruction of lysine are probably misleading, however, since as shown in a later part of this paper, it was impossible to *isolate* lysine from Deaminized Casein VIII, which showed by the Van Slyke procedure a lysine nitrogen content of 2.68 per cent.

On the whole, the results obtained by the Van Slyke partition method closely resemble those previously obtained by one of us (1) and do not indicate that, under the experimental conditions used, the temperature of deamination played an important rôle in influencing the composition of the deaminized product.

Tyrosine and Tryptophane Content of Deaminized Casein.

Early in the study of the composition of deaminized proteins, it was observed that a part at least of the tyrosine was destroyed or modified in some way by the reagents used in deamination (3). The few quantitative studies of the tyrosine content of deaminized casein have been made by the use of the Folin-Denis colorimetric method (1) or some modification (3) and have indicated a lowered chromogenic value with the phenol reagent after deamination. Steudel and Schumann (8), in a recent paper published after the completion of the present study, have been unable to observe any change in either the tyrosine or tryptophane content of casein deaminized according to the procedure of Dunn and Lewis (1) (as determined by the method of Folin and Looney).

The possibility of reactions other than the replacement of the amino group has long been recognized. Treves and Salomone (9) because of the yellow color of their products and their reactions to certain tests for diazo compounds were led to postulate the formation of diazo derivatives. Dunn and Lewis (1) suggested nitroso derivatives of the various cyclic amino acids as a possible explanation of the pigmentation.

Morel and Sisley have recently studied the reaction of nitrous acid on silk (10) and upon tyrosine (11) at low temperatures (below 10°), and believe that they have secured evidence of diazotization of part of the tyrosine. Evidently under the conditions employed by them, only a portion of the tyrosine was deaminized to form *p*-hydroxy-phenyllactic acid. They were unable to obtain complete diazotization of the tyrosine, a maximum of 70 per cent being reached in about 36 hours. The diazo derivatives of tyrosine and its products of deamination, unstable at ordinary temperatures, yielded on reduction (stannous chloride or sodium hydro-sulfite) the corresponding 3-amino derivatives. These on treatment with nitrous acid gave the same color as was obtained by prolonged treatment of tyrosine with nitrous acid. They suggested that in the reaction of nitrous acid on silk, nitroso groups first entered the tyrosine molecule in a position ortho to the hydroxy group. These nitroso groups were then reduced to amino groups and the primary amine group thus formed was diazotized.

In our study we have used the methods for the determination of tyrosine and tryptophane described by Folin and Ciocalteu (12). Difficulty was experienced, however, in applying the modified Millon reaction to the hydrolysates of deaminized casein.

Analyses were made on three types of hydrolysates prepared as outlined below.

1. *Acid Hydrolysate*.—1 gm. of protein was hydrolyzed for 18 hours with 20 cc. of 20 per cent hydrochloric acid and the excess acid was removed by evaporation on the steam bath under diminished pressure, 9 cc. of 14 N sulfuric acid were added, and the solution made up to 100 cc. in a volumetric flask.

2. *Barium Hydroxide Hydrolysate*.—1 gm. of protein was boiled with 4 gm. of barium hydroxide and 25 cc. of water for 48 or 96 hours. To the hot solution were added 30 cc. of 20 per cent sulfuric acid and the flasks were heated in a boiling water bath for 30 to 40 minutes to remove any hydrogen sulfide present. The solution was then diluted to 100 cc. and filtered.

3. *Sodium Hydroxide Hydrolysate*.—1 gm. of protein was hydrolyzed as described by Folin and Ciocalteu (12).

The tyrosine and tryptophane were separated as described by Folin and Ciocalteu (12) except that only one-half the amounts

of reagents and sample specified by them were used with a correspondingly weaker standard. After the last washings of the tryptophane precipitate had been transferred to the volumetric flasks, 30 cc. of water, 2 drops of caprylic alcohol, and 25 cc. of saturated sodium carbonate solution were added in the order named to each flask and to the standard. The flasks were then shaken and 4 cc. of a 5 per cent sodium cyanide solution were added. The flasks were shaken until the precipitated mercuric oxide was dissolved and 5 cc. of the phenol reagent of Folin and Ciocalteu were added, the solutions after thorough mixing were allowed to stand 10 to 30 minutes, diluted to 100 cc., and the color comparisons made.

As already pointed out, Morel and Sisley (11) have observed that to the phenol group of tyrosine after treatment with nitrous acid, a diazo group was added and that this diazo group could be reduced to the corresponding amine. Since it seemed likely that the amino group might be less effective in the reduction of the phenol reagent of Folin than the diazo group, an attempt was made to reduce the hydrolysates and to compare the chromogenic values before and after reduction. Aliquots of the hydrolysates were reduced for 2 to 3 hours by the addition of a little zinc, the acid of the hydrolysates being sufficient to liberate hydrogen by its reaction with the zinc. It was hoped in this way that color development other than that resulting from the phenol group might be avoided. Analyses were then made on the reduced solutions, 4 cc. aliquots being used as already outlined. The original hydrolysates are designated as "unreduced" in Table II while the values obtained after treatment with zinc are presented in the column headed "reduced."

The results of the analyses of casein and deaminized casein by the modified Millon reaction of Folin and Ciocalteu are not presented in tabular form. The figure obtained for the tyrosine content of casein (6.51 per cent) is in good agreement with the values reported by Folin and Ciocalteu (12). It was impossible to obtain satisfactory results when this method was applied to the hydrolysates of deaminized casein, since the faint red color obtained could not be satisfactorily compared with the standard color, due to the yellow tinge in the hydrolysate. However, the color produced was probably less than half that obtained with

an equal amount of casein. The color developed in the modified Millon reaction was not significantly affected by the reduction of the hydrolysates with zinc and sulfuric acid.

A comparison was made of the reaction of a number of phenols in the Millon test as ordinarily applied and as modified by Folin and Ciocalteu. Tyrosine and phenol were the only phenols of those studied which gave positive tests by the modified test, although all the others (vanillin, salicylic acid, catechol, resorcinol,

TABLE II.
Tyrosine Content of Casein and Deaminized Casein (Modified Method of Folin and Looney).

Hydrolytic agent.....	HCl 18 hrs.		NaOH 18 hrs.		Ba(OH) ₂	
	Unreduced.	Reduced.	Unreduced.	Reduced.	Unreduced.	Reduced.
Treatment of hydrolysate.....						
	per cent	per cent	per cent	per cent	per cent	per cent
Casein II.....	6.44	6.35	6.56	6.38		
Deaminized Casein V*.....	6.45	6.50	5.98	6.43		
“ “ VI.....	5.33	5.39	5.65	5.75		
“ “ VII.....	4.67	5.13	5.59	6.08		
“ “ VIII.....	4.73	4.70	4.91	5.53	3.80†	4.63†
“ “ A 64†.....	4.82	5.56	5.68	6.05	4.43†	4.62†
					4.39§	4.52§

* See Table I for characterization of the various samples of deaminized casein.

† Hydrolyzed for 48 hours.

‡ This is the deaminized casein used by Dunn and Lewis (1).

§ Hydrolyzed for 96 hours.

hydroquinone, 3, 4-dioxyphenylalanine, and phloroglucinol) gave positive tests with the original Millon test. Phenol, compared with an equivalent amount of tyrosine, gave by the Folin-Ciocalteu procedure only about 70 per cent of the color developed by tyrosine. From the different behaviors of these substances in these two tests it seems that the reaction as outlined by Folin and Ciocalteu is more specific than the original Millon test and that color production is inhibited by the presence of substituent groups on the phenol nucleus.

The results obtained with the modification of the Folin-Looney method as described were more satisfactory (Table II). In the two proteins hydrolyzed with both sodium and barium hydroxide, the figures obtained with the sodium hydroxide hydrolysates were higher than those with the barium hydroxide hydrolysates, a result in accord with the work of Folin and Ciocalteu (12) who found lower values in the case of barium hydroxide hydrolysates. The hydrolysates of the deaminized caseins after reduction usually gave higher values than before reduction, especially in the case of the sodium hydroxide hydrolysates, where the values often approached those obtained for casein. However, in most cases, particularly with the deaminized caseins¹ prepared at room or higher temperatures (Deaminized Caseins VI to VIII, Table II), there appeared to be a decrease in tyrosine as compared with the values for casein.

The question is again raised as to whether this apparent change represents an actual destruction of tyrosine or the inhibition of color production by the presence of some substituent group on the phenol nucleus. An attempt was made to remove any nitroso groups if present by hydrolyzing with 20 per cent hydrochloric acid for 18 hours. The results obtained with these acid hydrolysates (Table II) were very similar to those obtained with the alkaline hydrolysates after reduction except in the case of Deaminized Caseins VII and VIII, where the tyrosine content was lower.

A quantitative study was made of the color production of various substituted phenols with the phenol reagent, using the color developed by tyrosine as a standard. The compounds used were dissolved to give solutions containing the molecular equivalent of 1 mg. of tyrosine per cc. Phenol and the substituted monophenols studied (vanillin, salicylic acid) gave less color than an equivalent amount of tyrosine. The diphenols (catechol, resorcinol, hydroquinone) gave more color than tyrosine but not the theoretical amount expected if both hydroxy groups reacted

¹ It is interesting to note that the values obtained for Deaminized Casein A 64 were similar to, although slightly higher than those previously reported (1). This sample of deaminized casein, prepared and previously analyzed in 1920, had been kept in a brown glass bottle for almost 10 years previous to the second analysis.

quantitatively. 3,4-dioxyphenylalanine more nearly approached the theoretical value than any other diphenol, giving 174 per cent of the color produced by the molecular equivalent of tyrosine. Phloroglucinol gave more color than tyrosine (121 per cent) but much less than the 300 per cent to be expected if all three hydroxy groups had reacted quantitatively.

These results seemed to indicate that the presence of another group on the benzene ring of phenol influences the reaction with the phenol reagent. In the light of these results, the proof that a part of the tyrosine in deaminized casein contains a second substituent group on the benzene ring would lend weight to the hypothesis that there is no actual destruction of the tyrosine molecule during deamination, but that the decreased chromogenic value with the phenol reagent is merely an expression of a diminished reactivity due to the presence of a substituent group in the tyrosine molecule. With this in mind, an attempt was made to isolate a derivative of tyrosine from the hydrolysate of Deaminized Casein VIII.

On the assumption that the most probable derivative formed in deamination would be either a nitroso or diazo derivative, the hydrolysate was reduced with zinc with the hope that a derivative more basic than tyrosine might be formed, a substance precipitable by some one of the alkaloidal reagents. 5 gm. of Deaminized Casein VIII were hydrolyzed with 20 gm. of barium hydroxide and 100 cc. of water for 48 hours. After the addition of an excess of sulfuric acid and the removal of the barium sulfate by filtration, the filtrate was reduced by adding zinc to the acid solution. The reduction was allowed to proceed for 3 hours at room temperature. After the removal of the sulfuric acid by the addition of a slight excess of barium carbonate and filtration, the filtrate was evaporated on the steam bath to a thick syrup, absolute alcohol added until a slight turbidity developed and an alcoholic solution of picric acid added. After standing overnight in the ice box a yellow needle-like precipitate was deposited. This was recrystallized from the minimum amount of water. This substance, after drying, melted at 256° (uncorrected). A micro analysis (Pregl) for nitrogen showed the presence of 17.18 per cent of nitrogen. The theoretical value for the nitrogen content of the monopicrate of monoamino tyrosine is 17.20 per cent. The

nitrogen contents of the picrates of the tyrosine itself or of the other amino acids found in the protein molecule do not fall within this range. An unsuccessful attempt to prepare aminotyrosine (13) for comparison was made. It is hoped that a further study may be made of this point.

From these results and in view of the work of Morel and Sisley, we do not consider that any evidence of the destruction of tyrosine in the sense that the molecule is disrupted has been obtained. The decreased color production is probably to be explained on the basis of the formation of a derivative of tyrosine during deamination with a lower chromogenic value with the phenol reagent than tyrosine. The results with the method of Folin and Ciocalteu indicated that a part only of the tyrosine was so affected. This would explain the isolation of tyrosine from the hydrolysates of deaminized casein by Dunn and Lewis (1). It is obvious that colorimetric methods for the determination of tyrosine are unsatisfactory under conditions in which the tyrosine may be modified by the addition of another group to the benzene ring.

The tryptophane contents of deaminized casein and casein were determined by both of the methods suggested by Folin and Ciocalteu. The value obtained for casein (1.30 per cent) was similar to the value obtained by them. The tryptophane content of four different deaminized caseins varied between 1.26 and 1.42 per cent, values closely similar to those obtained for casein, and undoubtedly within the range of experimental error of the method. The failure of deamination to affect the chromogenic value of casein in the tryptophane determination is in accord with the results of Steudel and Schumann (8), who also observed no change in the tryptophane content of deaminized casein as compared with casein.

Basic Amino Acids of Deaminized Casein.

Vickery and his coworkers have recently revised and extended (14, 15) the Kossel method for the separation and quantitative isolation of the basic amino acids of the protein molecule. Their procedure has also been modified by Calvery (16) to make the method available for the analysis of relatively small amounts of protein. Inasmuch as the data concerning the basic amino acids of deaminized proteins are confused and have been obtained by

the older methods, an analysis of Deaminized Casein VIII by the newer methods has been undertaken. After the completion of the work, a similar study of deaminized casein in which methods different from those employed by us were used was reported from the laboratory of Steudel (8).

In general we have followed the technique² described by Calvery (16). After the separation of the histidine by precipitation as the

TABLE III.
Basic Amino Acids of Casein.

The results are expressed as percentages of the total N of the protein. Each of the three analyses was made on a separate hydrolysate.

	Anal- ysis 1.	Anal- ysis 2.	Anal- ysis 3.	Aver- age.	Cal- very (16).*
	per cent	per cent	per cent	per cent	per cent
Histidine fraction (precipitated by mercury).					
Total N.....	3.28	3.05	3.24	3.19	3.43
Hanke-Koessler method (colorimetric)....	3.50	3.36	3.36	3.41	3.51
Plimmer-Phillips method (bromination)....	3.78	3.97	3.48	3.74	
Isolated as flavianate.....	2.11	2.38	2.16	2.22	3.01
Arginine fraction.					
Total N.....	8.64	8.68	8.73	8.68	9.34
N from alkaline hydrolysis.....	6.55	6.48	6.52	6.52	6.79
Isolated as flavianate.....	6.10	6.30	6.26	6.22	6.21
Lysine fraction.					
Total N.....	10.30	9.93	10.70	10.30	10.94
Isolated as picrate.....	4.72	5.33	5.60	5.22	6.49

* These figures are those obtained by Calvery on a hydrolysate from 50 gm. of casein ((16) p. 639, Table I, and p. 640, Table II, Experiment 5).

mercury salt, we have made use of various methods to determine histidine in the solution obtained after the removal of the mercury from the mercury-histidine precipitate; *i.e.*, colorimetric (Hanke-Koessler), bromination (Plimmer-Phillips), and isolation as flavianate. Similarly in the arginine fraction, we have determined arginine by alkaline hydrolysis and by isolation as the flavianate.

² We wish to express our indebtedness to Dr. H. O. Calvery for advice in the use of the procedure for the separation of the basic amino acids.

In the lysine fraction an attempt has been made to isolate and weigh lysine picrate. In all cases, 25 gm. samples of protein have been used for the analyses. The casein from which the deaminized casein was prepared was also analyzed. Fortunately the casein used for analysis by Calvery (16) was the same material so that a comparison of the results obtained by two different investigators on the same sample is possible.

The results obtained with casein are presented in Table III, together with the results obtained by Calvery. All of our own figures are in good agreement with those of Calvery with the possible exception of the values calculated from the weight of histidine isolated as flavianate. The values obtained by Calvery are probably more accurate since a larger sample of casein (50 gm.) was used for analysis and consequently a more complete precipitation of the rather soluble histidine diflavianate should have resulted. The figures obtained by bromination and by the colorimetric method are slightly higher than would have been expected from the total nitrogen of the fraction. It is possible that the higher value obtained by bromination is to be explained by the presence of traces of tyrosine or cystine in the solution, since the former requires 4 and the latter 16 atoms of bromine for complete reaction (17).

In contrast to the results by the Van Slyke procedure (Table I) as already discussed, which indicated the presence of more histidine in deaminized casein than in casein, the isolation method showed a decreased histidine content of casein after deamination (Table IV). The values for casein and deaminized casein were both lower than those reported by Skraup (18), but showed about the same relative decrease. Skraup found 2.6 and 1.18 per cent of histidine in casein and deaminized casein, while the figures presented here, when calculated on the basis of the per cent of histidine in these proteins, represent 1.75 and 0.78 per cent in casein and deaminized casein respectively. This decrease might have been due to the actual destruction of the imidazole ring under the prolonged influence of nitrous acid. Another possible explanation would be the formation of a substituted histidine derivative of decreased basicity which was not precipitated under the same conditions as was histidine. No evidence in support of either of these hypotheses is available.

The arginine content of casein (Table III), based on the total nitrogen of the fraction, was very similar to the value obtained by the Van Slyke partition method (Table I). The results obtained on hydrolysis of this fraction with sodium hydroxide, however, would indicate that this fraction was not made up of arginine alone. The values obtained by isolation of the arginine as the

TABLE IV. •
Basic Amino Acids of Deaminized Casein VIII.

The results are expressed as percentages of the total N of the protein. Each of the analyses was made on a separate hydrolysate.

Analysis No.....	1	2	Aver- age.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Histidine fraction (precipitated by mercury).			
Total N.....	1.63	1.29	1.41
Hanke-Koessler method (colorimetric).....	1.45	1.28	1.37
Plimmer-Phillips method (bromination).....	2.15	2.00	2.07
Isolated as flavianate.....	1.00	0.93	0.97
Arginine fraction.			
Total N of arginine fraction.....	1.77	6.50	
N from hydrolysis of arginine fraction.....	1.77	3.01	
" " " " lysine fraction.....	4.88	3.55	
Total arginine N by hydrolysis.....	6.65	6.56	6.61
Isolated as flavianate from arginine fraction....	1.84	2.98	
" " " " lysine fraction.....	1.42	3.26	
Total arginine N from isolation as flavianate..	3.26	6.24	
Lysine fraction.			
Total N	12.11	12.41	12.26
N of lysine fraction after correction for arginine			
N obtained by hydrolysis.....	7.23	8.86	8.04
Isolated as picrate.....	0.00	0.00	0.00

flavianate were not as constant as those obtained on alkaline hydrolysis, but represent minimum values, values which are probably close to the true value of the arginine content of casein, since the insolubility of the arginine silver compound and the arginine flavianate make this determination quite accurate. The slightly higher values obtained by alkaline hydrolysis may be due in part to the decomposition of other nitrogenous substances with the liberation of ammonia.

The arginine fraction of deaminized casein (Table IV) indicated a marked decrease of this amino acid as a result of the action of nitrous acid. Examination of the lysine fraction afforded evidence that this apparent decrease in arginine content was the result of incomplete precipitation of the silver salt of arginine, since on alkaline hydrolysis of the lysine fraction, an amount of nitrogen reacting as arginine nitrogen equivalent to 3 to 4 per cent of the total nitrogen was found. The sum of the values obtained by the hydrolysis of the lysine and arginine fractions with sodium hydroxide was approximately the same as the value found for casein by this method. That the substance present in this lysine fraction which reacted as arginine on alkaline hydrolysis was actually arginine, was proved by the isolation of arginine as the insoluble flavianate as shown in Table IV. In one analysis more than half of the total arginine was isolated as flavianate from the lysine fraction, and the total arginine isolated from the two fractions checked well in this determination with the values obtained for casein. In a second determination, the total arginine as isolated was about half of the value for casein. The appearance of a part of the arginine in the lysine fraction³ seemed to be of constant occurrence. No difficulty was experienced in effecting a separation of the arginine as the silver salt from the casein hydrolysate, but its precipitation from the hydrolysates of deaminized casein was never complete. The explanation of this is not clear. It is possible that it might have been due to some slight change in the arginine molecule which affected the solubility of its salts or more probably to the interference of some other substances produced in deamination which influenced the solubility of the arginine-silver compound.

The content of lysine in deaminized casein is of greater interest in this study than that of the other amino acids since if the current theory of the position of lysine in the protein molecule is correct, it should have been possible to remove quantitatively the ϵ -amino group of lysine by nitrous acid. The total nitrogen of the lysine fraction of casein (Table IV) agreed well with the values

³ It would be advantageous to confirm by further studies the presence of arginine in the lysine fraction of deaminized casein by the use of a more specific method of determination; as for example, the arginase method of Hunter and Dauphinee (19).

found for casein by the Van Slyke procedure (Table I). It should be noted, however, that only slightly more than half of this nitrogen could be isolated as lysine picrate. Leavenworth (20) from the hydrolysate from 1865 gm. of casein was able to isolate 5.77 per cent of lysine as the picrate. This is equivalent to about 7.4 per cent of the total nitrogen of the casein. This may be compared with the figure of 6.49 per cent reported by Calvery (16) and 8.70 and 9.36 per cent by Van Slyke (21). In the latter study, however, the procedure used (Kossel) did not involve actual isolation of the lysine as picrate. Probably the results of Leavenworth are the most accurate because of the large sample of casein hydrolyzed and the repeated crystallization from the mother liquors.

In an attempt to determine what portion of the total nitrogen of the lysine fraction was due to the presence of other nitrogenous substances, an aliquot of the lysine fraction from the casein hydrolysate was subjected to extraction with butyl alcohol for 15 hours, in accordance with the method of Dakin (22). If all the histidine and arginine had been precipitated before the addition of phosphotungstic acid, lysine should have been the only amino acid remaining, which was insoluble in butyl alcohol. About one-third of the nitrogen present was extracted by the alcohol and the residue after the extraction contained nitrogen equivalent to 6.91 per cent of the total nitrogen of the casein used. This checks fairly well with the figure reported for direct isolation by Leavenworth (20) and probably is close to the true value for the lysine nitrogen present in casein.

The total nitrogen content of the lysine fraction of the deaminized casein (Table IV) was much greater than was anticipated, but as previously stated this was due in part to a failure of complete precipitation of the arginine, which resulted in the appearance of arginine in the lysine fraction. After the correction for the arginine content of the lysine fraction was made, the total nitrogen indicated a destruction of about one-fifth of the lysine during deamination. Since no lysine could be isolated as the picrate, this residual nitrogen must be attributed to the occurrence of other amino acids in the lysine fraction. The failure to isolate lysine from the products of hydrolysis of deaminized casein is in accord with the findings of Levites (23).

In order to demonstrate further the absence of lysine in this fraction an aliquot portion of the lysine fraction of Analysis 1 (Table IV) was extracted for 15 hours with butyl alcohol. The liquid after extraction contained nitrogen equivalent to 4.3 per cent of the total nitrogen of the protein used. Since it has already been shown by alkaline hydrolysis and by direct isolation that this fraction contained about 4.88 per cent of the total nitrogen of the protein molecule in the form of arginine, and since arginine is not extracted by butyl alcohol, this would offer further evidence of the entire destruction of lysine in deamination. About 60 per cent of the nitrogen of the lysine fraction or 6.0 per cent of the total nitrogen of the protein was extracted by the butyl alcohol indicating the presence of monoamino acids in the lysine fraction.

SUMMARY.

1. Casein deaminized by the action of sodium nitrite and acetic acid (1) at various temperatures has been prepared and the deaminized casein analyzed by some of the newer methods of protein analysis.

2. Evidence of the inaccuracy of the Van Slyke method for the determination of characteristic groups of the protein molecule as applied to deaminized casein has been presented.

3. The arginine content of deaminized casein as determined by the Van Slyke procedure was slightly lower than that of casein. This was not substantiated by the results of the Vickery-Leavenworth method of analysis (14), by which practically the same amounts of arginine could be isolated as the flavianate from casein and deaminized casein.

4. The Vickery-Leavenworth method indicated a destruction of more than half of the histidine present in casein in the reactions involved in deamination.

5. The Van Slyke procedure indicated a degree of destruction of lysine varying with the temperature of deamination, while by the Vickery-Leavenworth method no lysine could be isolated from the hydrolysate of casein deaminized at 20°.

6. The tryptophane content of casein, as determined by the colorimetric method of Folin and Ciocalteu was not altered by the reactions of deamination.

7. Tyrosine as determined by the colorimetric methods of Folin

and his associates, appeared to be partially destroyed in deamination. Evidence has been presented to indicate that this decrease was probably due to the formation of derivatives of tyrosine during deamination, which had less chromogenic value in the color reactions used in the determination of tyrosine than tyrosine itself.

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